Isolation and bioinformatics analysis of the NADC30-Like CJS01 strain of the porcine reproductive and respiratory syndrome virus

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Veterinaria Italiana 2022, **58** (1), 47-55. doi: 10.12834/VetIt.2182.14564.1
Accepted: 09.09.2021 | Available on line: 16.11.2022

Keywords
Bioinformatics analysis,
Isolation,
PRRSV NADC30-Like.

Summary
In this study, lung tissue was collected from nursery piglets suspected of being infected with porcine reproductive and respiratory syndrome virus (PRRSV) in a large-scale pig farm in Sichuan, China. Polymerase chain reaction (PCR) and reverse transcription quantitative-polymerase chain reaction (RT-qPCR) methods were used to ensure that no other pathogens were present. Virus isolation was also carried out where the presence of PRRSV was determined by indirect immunoinfluscent assay (IFA). Compared with the common PRRSV strain, the isolate did not produce evident Cytopathic effect (CPE) in the early stage of isolation. CPE was found in the late stage, and the titer was $10^{4.17}$ $TCID_{50}$/0.1 mL. The strain was named CJS01. Bioinformatics analysis showed that it was a NADC30-Like strain. The virus load was determined by measuring the nucleic acid load during the proliferation of the strain on Marc-145 cells. The strain showed good adaptability on cells, and the virus proliferated on cells for 84 hr when the highest nucleic acid load was achieved. By recombinant analysis of ORF3~7 genes and prediction of its epitope, it was found that CJS01 strain might interfere with the immunesystem of the infected animals.

Introduction

Porcine reproductive and respiratory syndrome is caused by porcine reproductive and respiratory syndrome virus (PRRSV) (Yin *et al.* 2016), which mainly causes severe reproductive disorders, growth retardation, respiratory symptoms, and high mortality (Gen *et al.* 2012). At the end of the 20th century, in the early stage of epidemic, it was an unknown disease at that time, so named as "mysterious disease of pigs" (Kappes *et al.* 2015). As a new virus, there was a serious lack of understanding of the epidemiology and immunology of PRRSV (Done 1996). PRRS has been found for more than 30 years. In 1990s, the pig industry in Europe, the United States and other places was in a period of vigorous development (Baltag *et al.* 2013, Li 2015), which provided the necessary conditions for pathogen transmission. In 1996, Chinese scholar Guo Baoqing and other scholars (Guo *et al.* 1996) successfully isolated a strain named CH-1a, and used serological detection to trace PRRSV that has been popular in China for a long time.

Pigs of all ages have different degrees of susceptibility to PRRSV. PRRSV can cause damage to multiple organs of the body and seriously affect the health of pigs. It was once called one of the most expensive diseases (Rubio *et al.* 2009). No matter the semen, embryo or animal body, they all can be used as the natural medium of PRRS diffusing, and they play a role in the ultra long distance transmission of the virus (Xu *et al.* 2009).

PRRSV belongs to the RNA virus of arteritis virus, with a total length of 15 kb and 10 ORFs (Yin *et al.* 1997). As early as 1996, it has been reported that PRRSV has the characteristics of code shifting, recombination and mutation (Chen *et al.* 2008). With the passage of time, it has evolved the coexistence of Highly pathogenic (HP) PRRSV, GM2/QYYZ, 1-7-4 RFLP, NADC30-Like and other virus strains. The NADC30-Like showed 131 aa discontinuous deletion (Forsberg *et al.* 2002). Comparing with the classical PRRSV strain (VR2332, CH-1a), the amino acids of Nsp2 protein of NADC30-Like isolate were

absent at 322-432 (111 aa), 481 (1 aa) and 504-522 (19 aa) (Li 2015), which is of great significance for PRRSV typing. During the evolution of PRRSV, some virulent strains, such as PRRSV TJ, HuNAN, Hn-1/06, NADC30-Like recombinant strain JL580 (Zhang *et al.* 2018, Giles *et al.* 2015) were produced. Among all subtypes, recombination often occurs in different hot spot genomic regions (Kappes *et al.* 2015, Hopper *et al.* 1992, Spilman *et al.* 2009), such as GP5, Nsp2 and other genes. After recovering from infection, the animal is protected from infection with homologous strains or strains with high homology (He 2012, Lager *et al.* 1997). However, due to the strong heredity and antigenic variation of PRRSV, no widely effective and cross protective preparedness measures have been developed (Duan 2011). It has been pointed out that GP5 plays an important role in neutralizing antibody production in humoral and cell-mediated immune response after vaccination or field virus infection (Vu *et al.* 2017, Hu *et al.* 2009).

Materials and methods

Materials and reagents

The lung tissue of piglets suspected to be infected with PRRSV were collected from a large-scale pig farm in Sichuan Province, China. The marc-145 cell line was preserved in the laboratory of Sichuan Agricultural University (Cheng du, China). *Escherichia coli* DH5α was purchased from Beijing Tiangen Corporation (Beijing, China). T-Vector pMD19 (Simple) was purchased from Takara Corporation (Tokyo, Japan).

DMEM medium (high sugar type) was purchased from GIBCO, California, United States of America. Fetal bovine serum was purchased from Sangon Biotech, Shanghai (China). Anti-PRRSV N protein monoclonal antibody was purchased from GeneTex, San Antonio, United States of America (USA). PrimeScript RT Reagent kit and Triton X-100 were purchased from Takara Corporation, Tokyo, Japan. Genomic DNA/RNA extraction kit, DNA molecular weight standard, DNA gel recovery and purification kit and Plasmid extraction kit were purchased from Tiangen Corporation, Beijing (China). AceQ® qPCR SYBR® Green Master Mix was purchased from Vazyme-innovation in enzyme technology, Nanjing, China. Ampicillin was purchased from Amresco, Houston, USA. 1.1×T3 Super PCR Mix was purchased from Tsingke biological technology, Chengdu (China).

NADC30-Like positive sample specific detection

First, Porcine circovirus (PCV), Porcine epidemic diarrhea virus (PEDV), Transmissible gastroenteritis virus (TGEV), Pseudorabies virus (PRV), and Classic swine fever virus (CSFV) were detected by specific polymerase chain reactions (PCR). NADC30-Like infected materials were tested by the PRRSV NADC30-Like SYBR Green I real-time quantitative PCR (RT-qPCR) as described by Xiang and colleagues (Xiang *et al.* 2020).

Sample processing

NADC30-Like infected material and other pathogenic (HP-PRRSV, PCV, PEDV, TGEV, PRV, CSFV) negative materials were aseptically ground, frozen and thawed several times, then they were centrifuged, and the supernatant was collected. After that, a penicillin and streptomycin solution was added. After above operations have been completed, culturing overnight at 37 °C. After the treatment was completed, the homogenate was centrifuged and filtered by using a filter plug. The filtrate was collected and stored at -80 °C for subculturing.

Virus isolation

Marc-145 cells were grew in 24-well culture plates at 37 °C in 5% CO_2 until they reached 70-80% confluence.Confluent monolayers were then inoculated with filtrated homogenates, then added. Consequently, cells were incubated in CO_2 constant temperature incubator for 2 hours. After incubation, homogenate was discarded, and dulbecco's modified eagle medium (DMEM) was added. The cells were then incubated for 4-5 days. If CPE occurs, wait for the pathological area of cells to reach 60%. The suspension was then recovered, freeze-thawed and centrifuged. After that, the supernatant was extracted and purified before subcultur to the plaque. The purified suspension was continuously subcultured up to 17 passages, and the presence nucleic acid of the virus was controlled every 3 passages. If there is no CPE for 10 passages, the eventual presence of nucleic acid was also tested. The sample was considered as negative after 4 negative results in a row.

Indirect immunofluorescence assay (IFA)

Cell monoalyers at 70%~80% confluence were fixed with Paraformaldehyde Fix Solution (PFA) at room temperature for 30 minutes. The PFA was washed repeatedly for three times. Then Triton X-100 was added and kept still for 30 minutes. After that bovine serum albumin (BSA) was added for blocking and at room temperature for 2 hours. Then the blocking solution was discarded and anti-N protein monoclonal antibody was added and kept overnight. The cells were washed repeatedly for three times. After that, fluorescein isothiocyanate was added avoiding light

for 20 minutes. After washing and sucking away the superfluous liquid, ECLIPSE TS100 was used to select the appropriate wavelength for fluorescence observation and recording. After washing and sucking away the superfluous liquid, ECLIPSE TS100 was used to select the appropriate wavelength for fluorescence observation and recording.

Determination of half infection amount of the virus

Marc-145 cells were cultured adjusting the cell concentration to 1.5 × 10^5/ml in 96 wells plate. After that, the viral suspension was inoculated when the monolayer was 80% confluent, and incubated at 5% CO_2 atmosphere at 37 °C. Sixteen wells were selected as the negative control group, and 8 dilution gradients (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) were set at the same time. Twenty wells were inoculated with each dilution, and the observation and record were made after incubation for 5 days. $TCID_{50}$ was calculated by Reed-Muench method (Benfield *et al.* 1992).

Cell attack and virus copy number detection

Marc-145 cells were prepared, the cell concentration was adjusted to 1.5×10^5/ml in 12 well plate, when the cell density grew to about 80%, inoculate the virus, and diluted virus solution was added into each well. Cells were cultured in CO_2 incubator. One hole was selected for cell control, and three repeated test groups were set up. The virus was collected at 12 hr intervals (11 groups in total) within 0-120 hr after the exposure, and each group took 300 μL suspension to extract RNA.

Preparation of cDNA

RNA was extracted. Complementary DNA was prepared, and stored at - 20 °C.

Table I. *Test primer design table of the Porcine reproductive and respiratory syndrome virus (PRRSV).*

Primer name	Primersequence (5′-3′)	Product Length (bp)	Annealing temperature (°C)
PRRSV-1F	AATCAGTCGCGGTAGTCG	1,360	56
PRRSV-1R	ACAGTACGTAGAATTGGCACTC		
PRRSV-2F	CAGTACCTCAGGTCAGGTGTT	1,062	58
PRRSV-2R	CAACCACTAAGGATCGCTGGG		
PRRSV-3F	TTGGCAATGTGTCAGGCAT	1,120	58
PRRSV-3R	GCAGGGGCCAGAATGTACTTG		
PRRSV-4F	CTCCAGATGCCGGTTGTGC	749	55
PRRSV-4R	ATTTCGGCCGCATGGTTC		

Draw the growth curve

We calculated the viral nucleic acid load of each time point in the virus solution with the same volume, and the virus proliferation curve was drew with the time in the x-axis and the nucleic acid load in the y-axis.

Amplification and bioinformatics analysis of some structural genes

Specific primers were designed for multiple NADC30-Like strains (Table I), based on the PRRSV NADC30 sequence downloaded from Genbank (accession number: JN654459). These were then created by the BGI Sequencing Co., Ltd. We amplified the ORF3-7 gene fragment of the NACD-like virus strain as described in previous studies with some modifications (Liao 2019), and sent the amplified product to the BGI Sequencing Co., Ltd for sequencing. Sequences were stitched together and used to generate an evolutionary tree for the reference strain (Table II). In addition to the recombination analysis, we also predicted the protein antigen epitope and conducted homology analysis as described in previous studies with some modifications (Zhang 2006).

All the bioinformatics analysis software used are listed below:

- DNAMAN v6.0 software for assembling the sequence;
- MEGA v5.1 software for generating maximum-likelihood (ML) trees;
- Simplot v3.5 software for recombination analysis;
- DNAStar software for predicting the protein antigen epitope.

Table II. *Porcine reproductive and respiratory syndrome virus strains used in the study.*

Strain	GeneBank	Area	Year
JXA1-P80	FJY548853	China	2008
TJ	EU86024	China	2006
GX1003	JX912249	China	2010
CH-1a	AY032626	China	1996
VR2332	U87392	USA	1992
BJ-4	AF331831	China	2000
MLV	AF159149	USA	1992
QYYZ	JQ308798	China	2012
GM2	JN662424	China	2012
MN184C	MN184C	USA	2008
FJY04	KP860910	China	2014
HENAN-HEB	KJ143621	China	2012
NADC30	JN654459	USA	2008

Results

Sample NADC30-Like SYBR Green qPCR test result

When tested for HP-PRRSV, PCV, PEDV, TGEV, PRV, and CSFV by PCR, no specific amplified bands were found. Using the PRRSV NADC30-Like SYBR Green I qPCR detection method, the sample had a specific amplification curve (Figure 1).

Cytopathy observation

The PRRSV NADC30-Like strain positive materials were inoculated in Marc-145 and incubated in CO_2 incubator at 37 °C for 4-5 days. The nucleic acid of the virus was detected by qPCR every 3 passages. The results showed that the nucleus expanded slightly and became darker from the third to ninth passage, and it was similar to normal cells. CPE began to break off in large area and adhered to the cell surface in the form of clumps or grapes starting from the 10th passage (Figure 2). The CPE mainly showed cell aggregation, shedding, irregular shape and strong refraction.

Indirect immunofluorescence

The cultured Marc-145 cells were fixed with 5% PFA and stained with anti-N protein mAb and fluorescein isothiocyanate, respectively. Marc-145 cells that received NADC30-Like strain showed specific fluorescence after 4 days of challenge (Figure 3). Specific green fluorescence around nucleus or aggregation was observed in group A, but no specific fluorescence was observed in negative control group B.

Detection of fluorescence quantitative method

The NADC30-Like virus viral nucleic acid was detected from the 4th generation to the 15th generation. The titer of the 15th generation of NADC30-Like virus was higher than that of the 4th generation. This indicated that the isolate could adapt to Marc-145 cell line.

Determination and comparative analysis of half amount of virus infection

The titer of the isolate on Marc 145 cell line was $10^{4.17}$ $TCID_{50}$/0.1 mL. The results showed that the virus titer was slightly lower than that of the normal isolates, and the virus isolation process did not show obvious CPE in the earlier stage, even though the incubation time was longer.

Drawing of growth curve

After inoculation, the virus solution was detected in each period, and the relative nucleic acid load was measured to draw the growth curve. The results showed that the isolates were in the period of concealment from 0 to 40 hr, entered the logarithmic period at 48 hr, and began to enter the plateau period from 40 to 84 hr, and reached the peak at 84 hr (Figure 4). The rapid decline in virus volume may be due to the release of RNA from the cells into the extracellular environment at 37 °C and the degradation of RNA enzymes at ambient temperature for a long time due to RNA virus characteristics. The virus was completely inactivated at 37 °C for 48 hr. It can be seen that stability has

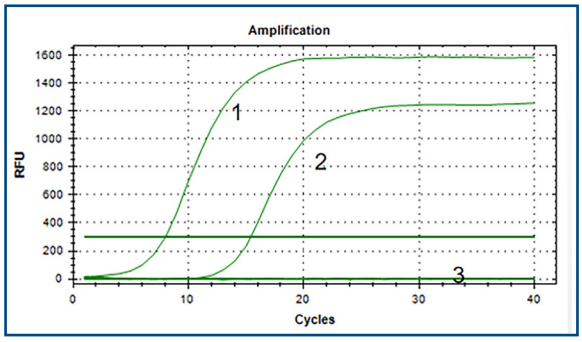

Figure 1. *qPCR test result of pig samples infected with Porcine reproductive and respiratory syndrome virus.*
1 = Positive control; 2 = Sample. 3 = Negative control.

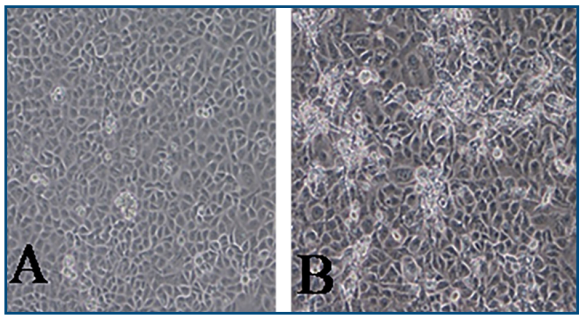

Figure 2. *Porcine reproductive and respiratory syndrome virus NADC30-Like CJS01 strain growth in Marc-145 cell line cytopathic effect.*
A. Negative control group. **B.** Infected group.

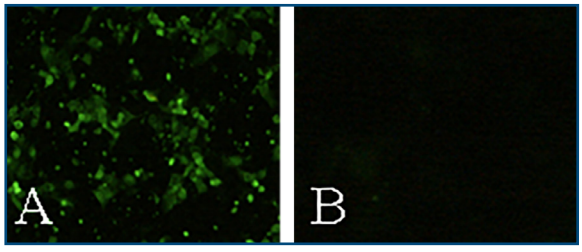

Figure 3. *Porcine reproductive and respiratory syndrome virus NADC30-Like CJS01 strain growth in Marc-145 cell line. Immunofluorescence test results.*
A. Infected group. **B.** Negative control group.

great influence on it, and it is also important to choose a suitable time for virus harvest.

Amplification of partial structural genes

According to the instructions of RNA extraction kit, viral nucleic acids were extracted. The target bands were located at 749 bp, 1,120 bp, 1,062 bp and 1,360 bp, respectively (Figure 5). According to the instructions of the gel recovery kit, the target conditions were recovered and ligated.

Bioinformatics analysis

The nucleotide sequence of ORF3~7 was obtained by splicing the entire sequence (2,566 bp). Two maximum-likelihood (ML) trees (Figure 6-A and Figure 6-B) of ORF5 and ORF3-7 gene fragments were constructed by bioinformatics analysis of BJ-4, JXA1 and TJ strains. The homology of ORF3 gene, ORF4 gene, ORF5 gene, ORF6 gene, ORF7 gene and ORF3~7 gene versus the NADC30 strain (JN654459) were 94.8%, 95.5%, 94.1%, 92%, 98.1% and 94.4%, respectively (Table IV). These results indicated that the GM2/QYZ type strain was also identified in the strains of other subtypes, which belonged to a type of recombinant strain, mostly HP-PRRSV and NADC30-Like.

Using DNAman and simplot software, partial structural gene analysis revealed that the fragment recombination between NADC30-like strain HENAN-HEB and FJY04 (Figure 6-E) occurred in CJS01 strain. The ORF5 and ORF6 genes were mainly located on the ORF5 and ORF6 genes, i.e. GP5 and M proteins. The ORF5 gene was located at 87~294 bp and 507~603 bp, and the accumulative length of the recombinant fragment was about 303 bp, accounting for 50.2% (303/603) of the ORF5 gene. The ORF6 gene was located at 459~489 bp. The accumulative length of ORF6 gene was about 45 bp, accounting for 8.6% (45/525) of the ORF6 gene. The recombinant gene accounted for 31.3% (348/1,112) of GP5 and M genes, and 13.6% (348/2,565) of ORF3~7 genes.

The ORF5 gene and ORF6 gene of PRRSV CJS01 strain were predicted by antigenic epitopes (Figure 6-C and Figure 6-D). The results revealed that there were potential epitopes at 87~294 (aa29~89), 507~603 (a189~201) and 1~141 (aal~47), 459~489 (a153~163) of ORF5 gene. This indicates potential epitopes in the recombination loci.

Table III. *Porcine reproductive and respiratory syndrome virus NADC30-Like CJS01 strain growth in Marc-145 cell line.* Number of wells showing cytopathic effect (CPE).

Dilution of stock solution	Number of wells with CPE holes	Number of wells without CPE	Cumulative		Percentage of CPE holes
			Number of wells with CPE	Number of holes without CPE	
10^{-1}	20	0	74	0	100% (74/74)
10^{-2}	20	0	54	0	100% (54/54)
10^{-3}	20	0	34	0	100% (34/34)
10^{-4}	12	8	14	8	60% (14/22)
10^{-5}	2	18	2	27	7% (2/29)
10^{-6}	0	20	0	47	0 (0/47)
10^{-7}	0	20	0	67	0 (0/67)
10^{-8}	0	20	0	87	0 (0/87)

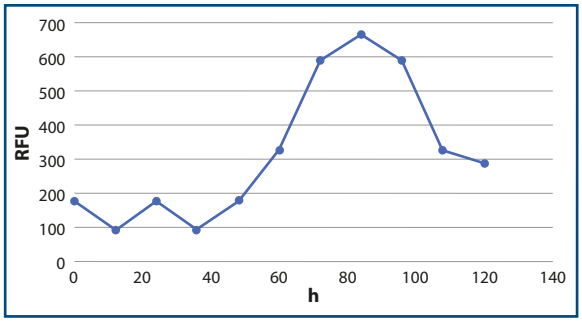

Figure 4. *Growth curve of the Porcine reproductive and respiratory syndrome virus NADC30-Like CJS01 strain in Marc-145 cell line. h:hours, RFU:Relative Fluorescence Unit.*

Discussion

In this study, NADC30-Like strain positive samples were grown on Marc-145 cells. A PRRSV NADC30-

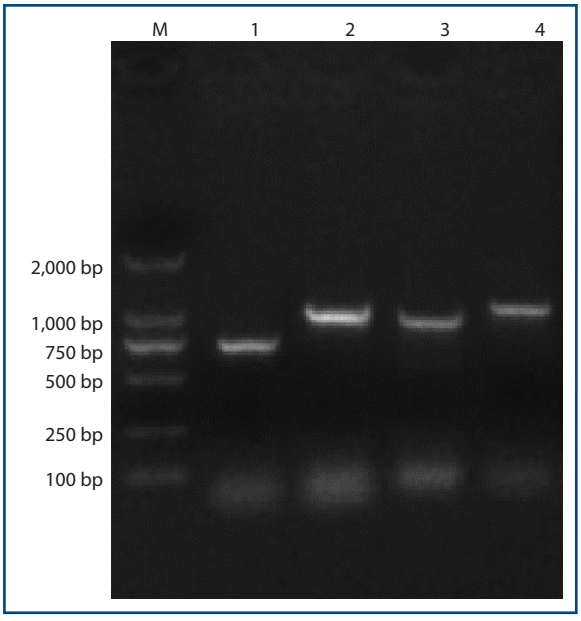

Figure 5. *RT-PCR amplification of CJS01 strain structural gene ORF3~7 fragment.* M = DNA Marker DL2000; 1~4 = Target segment.

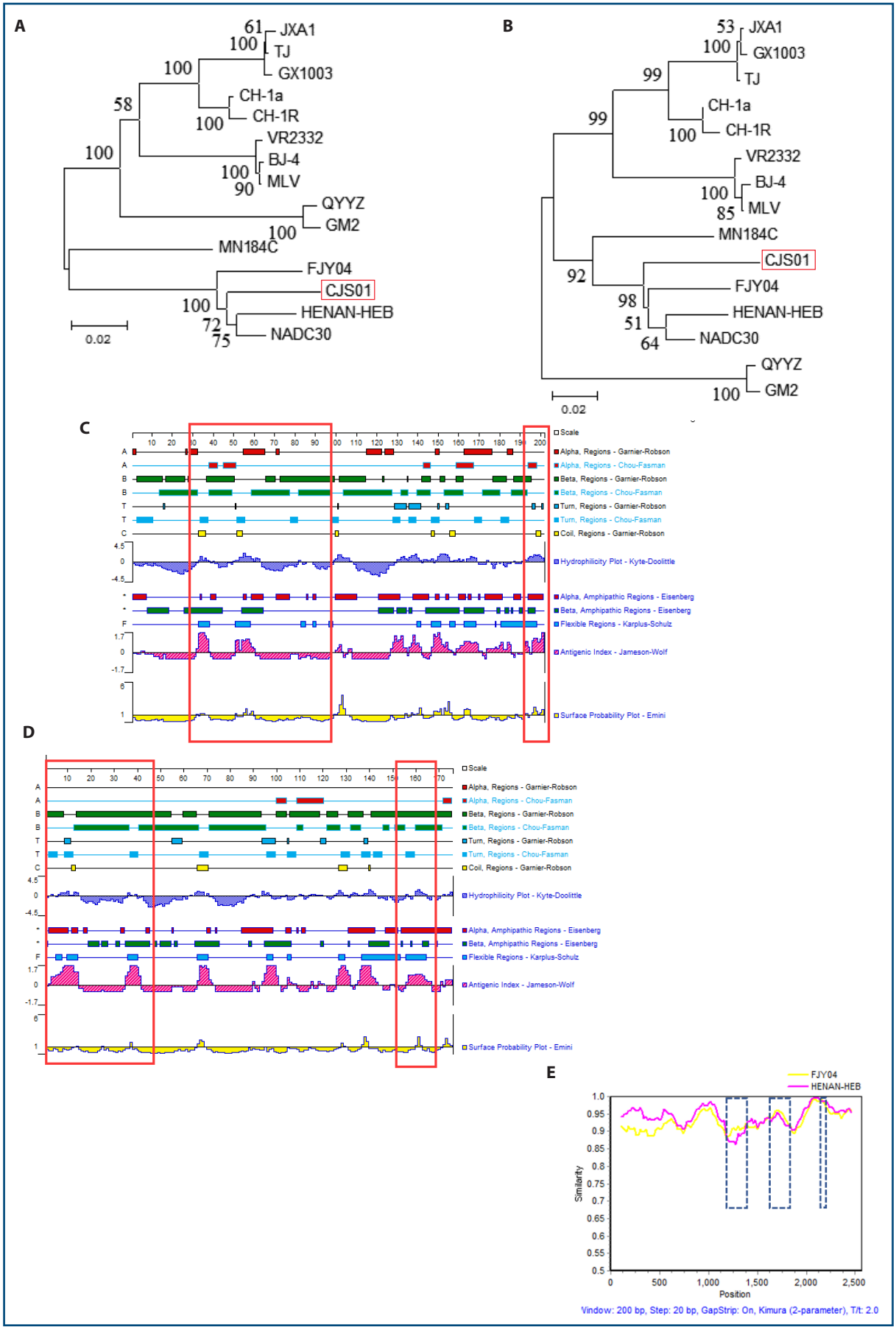

Figure 6. A. *Porcine reproductive and respiratory syndrome virus NADC30-Like CJS01 strain: ORF3~ORF7 structural gene phylogenetic tree.* **B.** *GP5 structural gene phylogenetic tree.* **C.** *GP5 epitope prediction.* **D.** *GP6 epitope prediction.* **E.** *Simplot analysis of ORF3~ORF7 recombination sites.*

Table IV. *Nucleotide homology analysis.*

Strain	ORF3	ORF4	ORF5	ORF6	ORF7	ORF3~ORF7
NADC30	94.8	95.5	92.9	96.8	98.1	84.5
BJ-4	82.2	85.7	84.9	89.7	91.9	86.3
MN184C	81.7	88.8	88.4	89.1	89.8	87
JXA1	81.7	84.5	85.9	89	90.3	93.3
GX1003	81.2	85.3	85.4	89	89.8	85.7
HENAN-HEB	94.4	95.2	91.9	95.6	96	85.5
FJY04	91.4	93.9	91.9	95.2	95.4	94.5
TJ	81.4	85.3	85.9	88.8	90.3	85.8
MLV	82.2	86	85.1	89.7	91.9	86.4
CH-1a	81.4	85.7	86.6	88	90.9	85.9
VR2332	82	86	85.1	89.9	92.5	86.4
QYYZ	81.4	84.5	86.1	88	90.9	84.6
CH-1R	80.5	85.7	84.1	88.8	87.6	84.4
GM2	80.8	85.1	83.4	89.3	85.8	95.4

Like strain with good adaptability was obtained. It was named PRRSV CJS01 strain. There was no obvious CPE in the early stage of virus passage up to the 10th passage when an obvious CPE was observed. The adaptability of the strain may be related to N protein. N protein, which accounts for about 35% of the total viral constituent proteins, is an important structural protein in virus assembly and is often enriched in nuclei and nucleoli. The Marc-145 cell's poly(A)-binding protein (PABP) specifically binds to the N protein. This combination inhibits cell protein synthesis and reduces virus replication, resulting in a decrease in virus titer in the cell. It was observed that the N protein surface gathered in the nucleus and cytoplasm of the infected Marc-145 cells showed a granular appearance. Therefore, it was speculated that the nucleus and nucleolus of the infected cells might have pathological changes during the early separation process, which could be one of the reasons for the failure of virus isolation. Among them, the variation of N gene in NADC30-Like PRRSV was located in the presence of antigenic epitopes, possibly leading to changes in the corresponding sites of specific recognition and binding of antigenic determinants. The qPCR method was used to detect the nucleic acid load in the process of virus proliferation. It was found that the nucleic acid content was the highest after 84 hr from Marc-145 cell infection. To obtain high titers of virus, collecting and passaging in this period of time would contribute to a good efficacy. Through the analysis of the biological characteristics of the isolated strain, it was found that the recombination mutation event also occurred in this strain, and the mutation rate of PRRSV was obviously accelerated in recent years. Hence, it is speculated that the antigen drift may not only be caused by gene mutation, but also caused by the recombination between strains, and the number of nucleotides in one mutation is obviously superior to that in gene recombination. The reason may be the joint action of the error correction mechanism of PRRSV itself and the external selection pressure. It is worth noted that the aa37-44 of GP5 is an important neutralizing epitope that determines the neutralization reactivity of serum. Aa102 and aa104 of GP5 play an important role in virus susceptibility (Sun 2016), and aa10 of GP6 has a great influence on immune neutralization. This study shows that the recombinant is mainly located on GP5 and M protein, and the fragment of the recombinant contains the production range of virus neutralizing antibody and neutralizing antibody, which has a direct relation to immune escape (Zhang *et al.* 2018). There is antibody-dependent enhancement (ADE) effect in PRRSV, and it has been proved that the amino acid region leading to this effect is also in this range. With the antigen drift of the strain, the ADE effect may follow this region into the recombinant strain, and the effect is also related to the parent strain. The previously reported PRRSVNADC30-like JL580 strain can produce stronger immunosuppression, which is speculated to be associated with this interval recombination. If the genes carrying stronger immunosuppressive regions, such as drug resistance genes, are transferred between strains, the harm may be more serious. Most of the recombination events of PRRSVNADC30-Like strain occurred with HP-PRRSV strain.

In TIAN K's study (Tian 2017), PRRSV NADC30-Like was compared with other strains (MN184 and SDSU73). Pigs infected with NADC30-Like had an early febrile and peak at 8 days post infection (dpi). NADC30-infected pigs developed earlier viremia which could be detected as early as 1 dpi. The NADC30-infected pigs developed interstitial pneumonia, but relatively milder as compared with SDSU73 and MN184. The above results suggested PRRSV NADC30-Like as relatively mild in virulence. In his study, a PRRSV NADC30-like strain showed much lower pathogenicity as compared with HP-PRRSV.

The harm caused by immunosuppression of HP-PRRSV strain among many PRRSV strains is the most serious. This study suggests that there are two important epitopes of PRRSV in this recombination interval, which control the production of immunosuppression and neutralizing antibodies, respectively. It is considered that this phenomenon is one of the causes of clinical immune failure. Through the prediction of the antigenic epitopes of the strain, it was revealed that all the segments of the recombination occurred in the presence of potential or confirmed antigenic epitopes. The occurrence of this recombination phenomenon is speculated to be caused by the huge pressure drive caused by the widespread use of PRRSV vaccine. Besides, improving

the detection methods of PRRSVNADC30-Like and strengthening the epidemiological investigation of PRRSV are of far-reaching significance for the scientific prevention and control of PRRSV.

Acknowledgments

This work was funded by the Special Project of Sichuan Veterinary Drug Innovation Team of National Agricultural Industry Technology System.

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